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International application number: PCT/US05/002697

International filing date: 31 January 2005 (31.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/540,896
Filing date: 30 January 2004 (30.01.2004)

Date of receipt at the International Bureau: 21 February 2005 (21.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
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February 09, 2005

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APPLICATION NUMBER: *60/540,896*
FILING DATE: *January 30, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/02697*

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Provisional Application Cover Sheet

Address to:
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This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. § 1.53(b)(2).

Docket Number: Q3426	Type a plus sign (+) inside this box	+

Inventor(s)/Applicant(s)

Last Name	First Name	Middle Initial	Residence (City and either State or Foreign Country)
Cappola Epstein	Thomas Jonathan		Haverford, PA Villanova, PA

Title of the Invention (280 Characters Maximum)

Novel Predictors of Cardiac Allograft Rejection Determine by Peripheral Blood Gene Expression Profiling

Correspondence Address

University of Pennsylvania
Center For Technology Transfer
3160 Chestnut Street
Suite 200

City: Philadelphia State: Pennsylvania Zip Code: 19104 - 6283 Country: US

Enclosed Application Parts *(check all that apply)*

[X] Specification *Number of pages:* 23 [ ] Small Entity Statement

[ ] Drawing(s) *Number of sheets* _____ [ ] Other (specify)

Method of Payment *(check one)*

[ ] Our Check No. _____ is enclosed to cover the Provisional filing fees. A duplicate copy of this sheet is enclosed. [X] The Commissioner is hereby authorized to charge filing fees and credit Deposit Account No. 13-2489. A duplicate copy of this sheet is enclosed. [ ] Payment by credit card. Form PTO-2028 is attached.	Provisional Filing Fee Amount ($)	$ 80.00

This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No
[ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Signature: _____ Date: 1/30/2004

Typed or Printed Name: Thomas Cappola

[ ] Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

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PROVISIONAL APPLICATION SUBMISSION TO USPTO – CONTENTS PAGE

Penn Docket Number	:	Q3426
First-named Inventor	:	Cappola
Submission Date	:	1/30/04
Prepared by	:	Matt Thomas

CONTENTS LISTED IN ORDER :

Total Number of Pages : __23__

Q3426/RBM

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UNIVERSITY OF PENNSYLVANIA
PRELIMINARY TECHNOLOGY DISCLOSURE FORM

PLEASE SEE REVERSE SIDE FOR INSTRUCTIONS

Date Submitted: December 22, 2003

1. **Disclosure Title.** Novel predictors of cardaic allograft rejection determine by peripheral blood gene expression profiling

2. **Relation to Previous Disclosure:** Yes ☐ No ☑ If Yes, file number and title: _____

3. **Possible Obligations to Others:**
Funding: NIH/Government ☑ Grant #: ME01370 Corporate or Other ☐ Sponsor Name _____

Related Agreements: ☐ Sponsored Research Agreements ☐ Collaborative Agreements ☐ Material Transfer Agreements ☐ Inter-Institutional Agreements

Other Parties (Include name/phone #, organization) _____

Materials: Did you use any material obtained from another party in developing this technology? Yes ☐ No ☑ Source: _____

4. **Critical Dates:**

	Circle One:	Date:	Describe:
-- Disclosure or presentation to others?	No **Yes**	11/10/2003	Who/Affiliation? American Heart Association
-- Submitted as an abstract or manuscript?	No **Yes**	5/30/2003	Expected Publication?
-- Submitted in grant application or report?	No Yes		Expected Funding?
-- Published in any form - including internet?	No **Yes**	10/28/2003	Where Published? Supplement to Circulation v.108(17).

Please include a copy of any such abstracts, manuscripts or grants with your Form.

5. **Commercialization:**
What products, processes or services would result from your technology? Blood test to predict, or exclude, cardiac transplant rejection
Do you know of (please provide names and contact information if possible):
Colleagues working in complementary areas? Yes. Expression Diagnostics (http://www.xdxinc.com). (privately held; a competitor)
Companies that might be interested in licensing your technology? Expression Diagnostics (http://www.xdxinc.com)

6. **Contributors:** I/We hereby submit this in accordance with University policies:

Signature(s)	Name (print)	Citizenship	School & Dept (or Institution if not Penn)	Phone #	Email
[Primary Contact]	Thomas ~~Capopla~~ Cappola	USA	School of Medicine	(215) 615-0805	thomas.cappola@uphs.
	Jonathan Epstein	USA	School of Medicine	(215) 573-9306	epsteinj@mail.med.upenn.edu
	Michael Parmacek	USA	School of Medicine	(215) 662-3140	michael.parmacek@uphs.
	Philip Horwitz	USA	Cardiovascular Division University of Iowa	(319) 353-6784	phillip-horwitz@uiowa.edu

7. **Description of Technology: (VERY IMPORTANT) CTT cannot assess the protectability, technical merit and commercial potential of your disclosure without this information.**

Please provide in hard copy and on electronic disk (IBM), if possible.
1) Grant applications and manuscripts describing the technology (as above).
2) Curriculum vitae (CV) of inventor(s).
3) Related publications and patents by you and others working in this field.
4) A concise description of the technology (2-5 pages), including the following:
   a) Brief Summary
   b) Stage of Development (Are there any problems with your present technology? Is there a need for additional funding, time, etc.?)
   c) Applications/Commercial use of the technology/Products or services envisioned
   d) Closest known similar technology or competing products
   e) Differences and advantages over other technology or products.

UNIVERSITY OF PENNSYLVANIA
PRELIMINARY TECHNOLOGY DISCLOSURE FORM
PLEASE SEE REVERSE SIDE FOR INSTRUCTIONS

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Funding: NIH/Government [✓] Grant #: ME01370 Corporate or Other [ ] Sponsor Name _____

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Materials: Did you use any material obtained from another party in developing this technology? Yes [ ] No [✓] Source: _____

4. **Critical Dates:**

	Circle One:	Date:	Describe:
-- Disclosure or presentation to others?	No **Yes**	11/10/2003	Who/Affiliation? American Heart Association
-- Submitted as an abstract or manuscript?	No **Yes**	5/30/2003	Expected Publication?
-- Submitted in grant application or report?	No Yes		Expected Funding?
-- Published in any form - including internet?	No **Yes**	10/28/2003	Where Published? Supplement to Circulation v.108(17).

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Colleagues working in complementary areas? Yes. Expression Diagnostics (http://www.xdxinc.com). (privately held; a competi
Companies that might be interested in licensing your technology? Expression Diagnostics (http://www.xdxinc.com)

6. **Contributors:** I/We hereby submit this in accordance with University policies:

Signature(s)	Name (print)	Citizenship	School & Dept (or Institution if not Penn)	Phone #	Email
[Primary Contact]	Thomas Capopla	USA	School of Medicine	(215) 615-0805	thomas.capopla@uphs.
	John Lepore	USA	School of Medicine	(215) 573-4774	john.lepore@uphs.upenn.edu

7. **Description of Technology: (VERY IMPORTANT) CTT cannot assess the protectability, technical merit and commercial potential of your disclosure without this information.**

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   c) Applications/Commercial use of the technology/Products or services envisioned
   d) Closest known similar technology or competing products
   e) Differences and advantages over other technology or products.

probeset	selected_up	selected_down	Title	Gene Symbol
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201454_s_at	FALSE	TRUE	aminopeptidase puromycin sensitive	NPEPPS
203388_at	FALSE	TRUE	arrestin, beta 2	ARRB2
204861_s_at	FALSE	TRUE	baculoviral IAP repeat-containing 1	BIRC1
211939_x_at	TRUE	FALSE	basic transcription factor 3	BTF3
208517_x_at	TRUE	FALSE	basic transcription factor 3	BTF3
210679_x_at	FALSE	TRUE	B-cell CLL/lymphoma 7A	BCL7A
211862_x_at	FALSE	TRUE	CASP8 and FADD-like apoptosis regulator	CFLAR
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205452_at	FALSE	TRUE	phosphatidylinositol glycan, class B	PIGB

215179_x_at	FALSE	TRUE	placental growth factor, vascular endothelial growth factor-related protein	PGF
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201174_s_at	TRUE	FALSE	telomeric repeat binding factor 2, interacting protein	TERF2IP
210598_at	FALSE	TRUE	transmembrane 6 superfamily member 2	TM6SF2
205849_s_at	TRUE	FALSE	ubiquinol-cytochrome c reductase binding protein	UQCRB

21 genes
12 ESTs

American Heart Association.
Fighting Heart Disease and Stroke
Scientific Sessions 2003, November 9-12, 2003, Orlando, Florida

Control/Tracking Number : 03-SS-A-12529-AHA
Activity : Abstract
Current Date/Time : 5/30/2003 4:35:56 PM

Novel Predictors of Cardiac Allograft Rejection Determined by Peripheral Blood Gene Expression

Phillip A Horwitz, Jonathan A Epstein, John J Lepore, Michael S Parmacek, Andrew C Kao, Shashank Desai, Lee R Goldberg, Mariell L Jessup, Thomas P Cappola; Hospital of the University of Pennsylvania, Philadelphia, PA

Endomyocardial biopsy is the gold standard for detecting cardiac allograft rejection, but is limited by invasiveness and cost. We tested the hypothesis that rejection could be detected by gene expression profiles in peripheral blood samples using oligonucleotide microarrays.
Methods: We performed a case-control study nested within a cohort of 189 cardiac transplant patients who had peripheral blood samples obtained during routine endomyocardial biopsy. Cases (n=4) of biopsy proven rejection (ISHLT grade 3A or 3B) were identified and compared to three different controls (n=4 in each group): paired samples from the same patients prior to rejection, paired samples after resolution of rejection, and unpaired samples from patients with negative biopsies. Labeled cRNA probes were produced from each sample and were hybridized to individual Affymetrix HU-133A oligonucleotide microarrays (16 in total). Expression data were analyzed using Robust-Multi-array Analysis and Significance Analysis of Microarrays algorithms.
Results: Of 22,000 transcripts assessed, 55 were differentially expressed in patients with rejection compared to pre- and post-rejection controls, with a false discovery rate <10% and change at least 2-fold in magnitude. The majority of these genes are involved in immune and inflammatory responses (31%), regulation of transcription or translation (20%), cell signaling pathways (18%) or cell growth and differentiation (11%). Further analysis demonstrated six transcripts that were differentially expressed in rejection compared to pre-, post-, and unpaired controls: soluble IL-1 receptor (GenBank accession U64094), mitochondrial superoxide dismutase (W46388), ras association domain family (NM_014737), TNF alpha-induced protein 2 (NM_006291), nuclear protein-tara (AF281030), and alpha 1-defensin (NM_004084).
Conclusions: These genes represent novel candidate predictors associated with the presence of cardiac allograft rejection at biopsy. If validated in larger patient cohorts, peripheral expression profiling may eventually allow post-transplant surveillance with blood testing rather than biopsy.

Commercial Relationship: P.A. Horwitz, None; **J.A. Epstein**, None; **J.J. Lepore**, None; **M.S. Parmacek**, None; **A.C. Kao**, None; **S. Desai**, None; **L.R. Goldberg**, None; **M.L. Jessup**, None; **T.P. Cappola**, None.

Category (Complete): Medical Management of Intrathoracic Transplantation
Additional Info (Complete):
Please select: : There are no unlabeled/unapproved uses of drugs or products.
Please select your preference of presentation: : Either
Male/Female: : Male
Ethnic Background: : Caucasian
AHA Member? : Yes
Please select: : Clinical Cardiology

Keyword (Complete): Transplantation/medical aspects ; Gene expression
Payment (Complete): Your credit card order has been processed on Friday 30 May 2003 at 2:39 PM.
Status: Complete

2003-12-22 Thomas, Cappola, M.D., Sc.M.

Novel Predictors of Cardiac Allograft Rejection Determined by Peripheral Blood Gene Expression

Phillip A Horwitz, Jonathan A Epstein, John J Lepore, Michael S Parmacek, Andrew C Kao, Shashank Desai, Lee R Goldberg, Mariell L Jessup, Thomas P Cappola; Hospital of the University of Pennsylvania, Philadelphia, PA

Concept: Endomyocardial biopsy is the gold standard for detecting cardiac allograft rejection, but is limited by invasiveness and cost. We tested the hypothesis that rejection could be detected nonivasively using periheral blood gene expression.

Progress Summary

First Analysis: In our first analysis, we performed a case-control study nested within a cohort of 189 cardiac transplant patients who had peripheral blood samples obtained during routine endomyocardial biopsy at the University of Pennsylvania. Cases (n=4) of biopsy proven rejection (ISHLT grade 3A or 3B) were identified and compared to three different controls (n=4 in each group): paired samples from the same patients prior to rejection, paired samples after resolution of rejection, and unpaired samples from patients with negative biopsies. Labeled cRNA probes were produced from each sample and were hybridized to individual Affymetrix HU-133A oligonucleotide microarrays (16 in total). Expression data were analyzed using Robust-Multi-array Analysis and Significance Analysis of Microarrays algorithms.

Of 22,000 transcripts assessed, 55 were differentially expressed in patients with rejection compared to pre- and post-rejection controls, with a false discovery rate <10% and change at least 2-fold in magnitude. The majority of these genes are involved in immune and inflammatory responses (31%), regulation of transcription or translation (20%), cell signaling pathways (18%) or cell growth and differentiation (11%). Further analysis demonstrated six transcripts that were differentially expressed in rejection compared to pre-, post-, and unpaired controls: soluble IL-1 receptor (GenBank accession U64094), mitochondrial superoxide dismutase (W46388), ras association domain family (NM_014737), TNF alpha-induced protein 2 (NM_006291), nuclear protein-tara (AF281030), and alpha 1-defensin (NM_004084).

We submitted these findings as an abstract to the 2003 American Heart Association Scientific Sessions on 5/3/03. These were accepted for oral presentation, which was given by Dr. Horwitz on 11/10/2003 in Orlando, Florida.

Second Analysis: In our second analysis, we compared peripheral blood expression profiles from 7 rejectors with 7 unmatched controls using the same approach as above. The larger sample size and simultaneous sample

hybridization with microarrays allowed for a more accurate analysis. We found 91 regulated genes with a false discovery rate < 10% that were associated with rejection.

We then looked at expressions profiles from the same 7 rejectors after they were treated and the rejection had resolved on biopsy (these samples are called "posts"). Interestingly, nearly all of the genes that were differentially expressed in the first comparison headed back toward the baseline level of expression in the controls, resulting in an intermediate expression profile for the posts. This is shown visually in Figure 1. Red indicates fold change in rejectors compared to control, and blue indicates fold change in posts compared to control. Nearly all the blue points are heading back toward the fold-change and are smaller in magnitude than the red points.

This is a significant finding. Using a resampling technique, we estimate the probability of finding this intermediate expression profile by chance is less than 1 in 10,000.

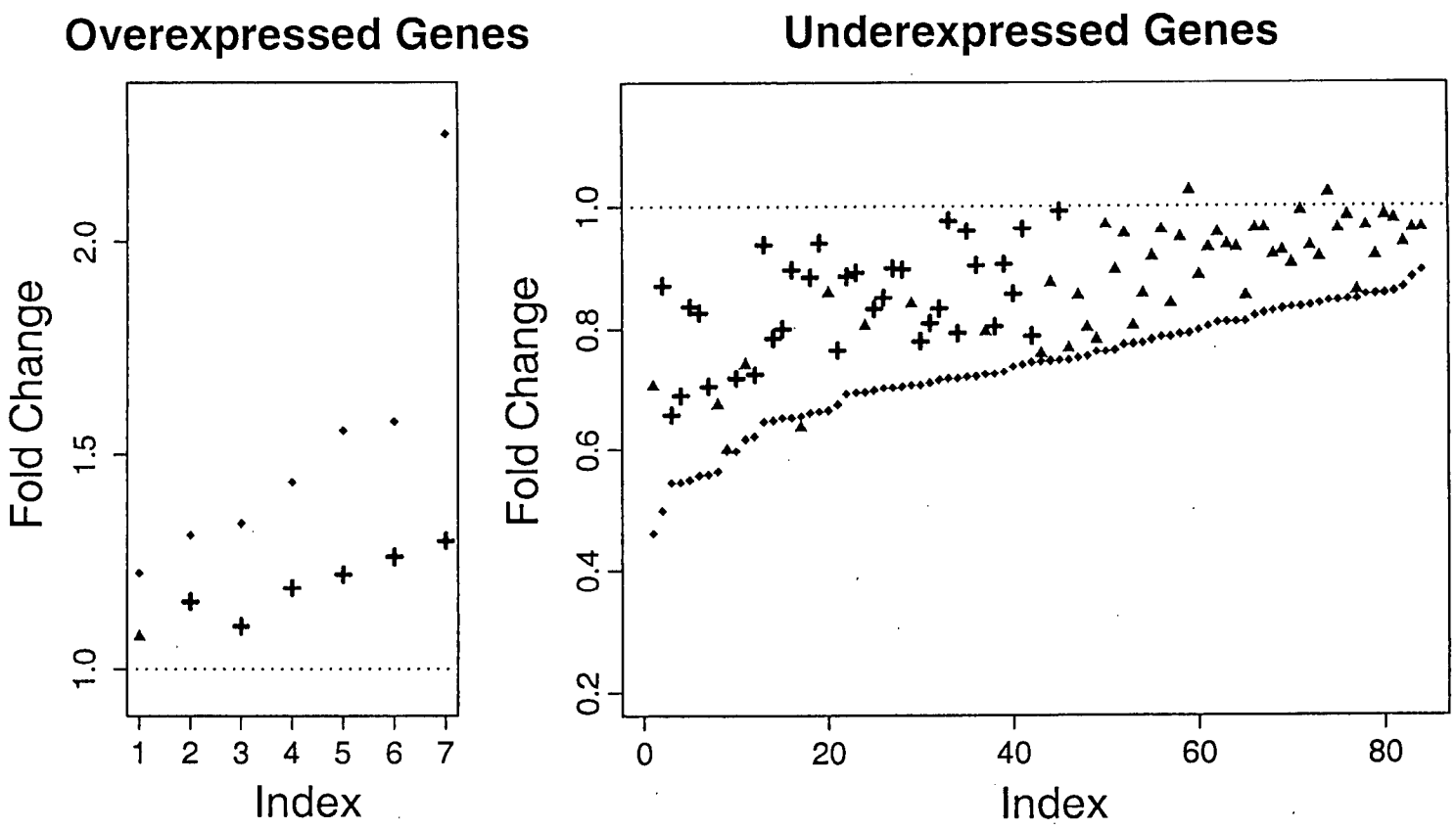
The intermediate expression profile of treated rejection is displayed another way in Figure 2 using hierarchical clustering. Using all 91 genes, there are two main branches in the dendrogram. One contains all the rejectors and the other contains all the controls. The posts are scattered between the two main branches.

Of the 91 genes, we chose 40 that showed the most consistent changes among all comparisons. These are listed at the end of this document using a variety of unique identifiers.

These are indicated by a cross in figure 1. Many of these are uncharacterized ESTs. However, 22 known genes popped up.

So thus far, we have demonstrated, in principle, that peripheral blood expression profiles correlate with solid organ rejection in a way that makes sense. These genes represent novel candidate predictors associated with the presence of cardiac allograft rejection at biopsy. If validated in larger patient cohorts, peripheral expression profiling may eventually allow post-transplant surveillance with blood testing rather than biopsy.

Next steps: Our next steps are 1) validation of our 91 candidates using quantitative PCR and 2) picking the best of these samples for propsective validation using new collected samples.


Overexpressed Genes
Fold Change
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1.5
2.0
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Index
Underexpressed Genes
Fold Change
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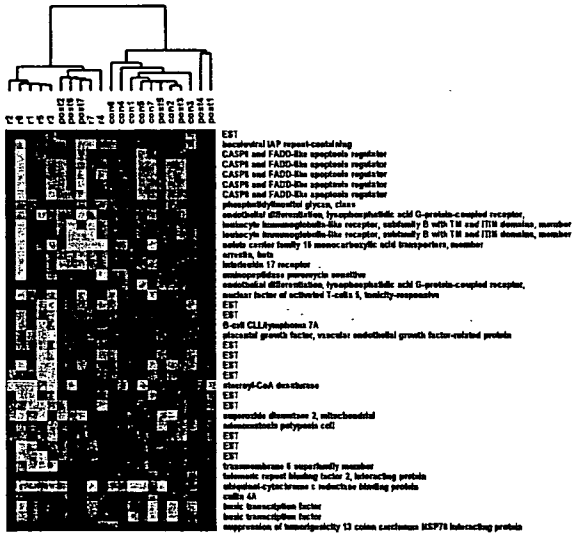
0.4
0.6
0.8
1.0
0 20 40 60 80
Index


EST
baculoviral IAP repeat-containing
CASP8 and FADD-like apoptosis regulator
CASP8 and FADD-like apoptosis regulator
CASP8 and FADD-like apoptosis regulator
CASP8 and FADD-like apoptosis regulator
CASP8 and FADD-like apoptosis regulator
phosphatidylinositol glycan, class
endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor,
leukocyte immunoglobulin-like receptor, subfamily B with TM and ITIM domains, member
leukocyte immunoglobulin-like receptor, subfamily B with TM and ITIM domains, member
solute carrier family 16 monocarboxylic acid transporters, member
arrestin, beta
interleukin 17 receptor
aminopeptidase puromycin sensitive
endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor,
nuclear factor of activated T-cells 5, tonicity-responsive
EST
EST
B-cell CLL/lymphoma 7A
placental growth factor, vascular endothelial growth factor-related protein
EST
EST
EST
EST
stearoyl-CoA desaturase
EST
EST
superoxide dismutase 2, mitochondrial
adenomatosis polyposis coli
EST
EST
EST
transmembrane 4 superfamily member
telomeric repeat binding factor 2, interacting protein
ubiquinol-cytochrome c reductase binding protein
colilin 4A
basic transcription factor
basic transcription factor
suppression of tumorigenicity 13 colon carcinoma HSP70 interacting protein

probeset	Title
216933_x_	adenomatosis polyposis coli
201454_s_	aminopeptidase puromycin sensitive
203388_at	arrestin, beta 2
204861_s_	baculoviral IAP repeat-containing 1
211939_x_	basic transcription factor 3
208517_x_	basic transcription factor 3
210679_x_	B-cell CLL/lymphoma 7A
211862_x_	CASP8 and FADD-like apoptosis regulator
210564_x_	CASP8 and FADD-like apoptosis regulator
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211317_s_	CASP8 and FADD-like apoptosis regulator
214486_x_	CASP8 and FADD-like apoptosis regulator
201423_s_	cullin 4A
206722_s_	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4
206723_s_	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4
216109_at	EST
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220071_x_	EST
205781_at	EST
215978_x_	EST
205707_at	interleukin 17 receptor
210784_x_	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3
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208003_s_	nuclear factor of activated T-cells 5, tonicity-responsive
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220232_at	stearoyl-CoA desaturase 4
221477_s_	superoxide dismutase 2, mitochondrial
207040_s_	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)
201174_s_	telomeric repeat binding factor 2, interacting protein
210598_at	transmembrane 6 superfamily member 2
205849_s_	ubiquinol-cytochrome c reductase binding protein

21 genes
12 ESTs

Gene Symbol	Map Locati	GO bio pro	GO cell coı	GO molec	GenMAPP	Unigene	OMIM	LocusLink
APC	5q21-q22	GO:7165;s	GO:5871;k	GO:8013;b	genmapp_	Hs.75081	175100	324
NPEPPS	17q21	GO:6508;p	GO:5634;n	GO:4177;aminopeptid		Hs.293007	606793	9520
ARRB2	17p13	GO:7165;arrestin;signal transduction;6.4e-54;				Hs.435811	107941	409
BIRC1	5q13.1	GO:6916;a	GO:5622;ir	GO:8189;apoptosis inl		Hs.79019	600355	4671
BTF3	5q13.3	GO:6355;r	GO:5634;n	GO:3702;RNA polyme		Hs.446567	602542	689
BTF3	5q13.3	GO:6355;r	GO:5634;n	GO:3702;RNA polyme		Hs.446567	602542	689
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CFLAR	2q33-q34	GO:6916;anti-apoptosi		GO:30693;caspase ac		Hs.355724	603599	8837
CFLAR	2q33-q34	GO:6916;anti-apoptosi		GO:30693;caspase ac		Hs.355724	603599	8837
CFLAR	2q33-q34	GO:6916;anti-apoptosi		GO:30693;caspase ac		Hs.355724	603599	8837
CFLAR	2q33-q34	GO:6916;anti-apoptosi		GO:30693;caspase ac		Hs.355724	603599	8837
CFLAR	2q33-q34	GO:6916;anti-apoptosi		GO:30693;caspase ac		Hs.355724	603599	8837
CUL4A	13q34	GO:82;G1/S transition of mitotic cell cycle;tra				Hs.270788	603137	8451
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						Hs.485406		
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IL17R	22q11.1	GO:7166;c	GO:5887;ir	GO:4872;receptor acti		Hs.129751	605461	23765
LILRB3	19q13.4	GO:6952;d	GO:5887;ir	GO:3824;catalytic acti		Hs.306230	604820	11025
LILRB3	19q13.4	GO:6952;d	GO:5887;ir	GO:3824;catalytic acti		Hs.306230	604820	11025
NFAT5	16q22.1	GO:6355;r	GO:5634;n	GO:3702;RNA polyme		Hs.86998	604708	10725
PIGB	15q21-q22	GO:6486;p	GO:5789;e	GO:3824;catalytic acti		Hs.259326	604122	9488
PGF	14q24-q31	GO:8283;c	GO:16020;	GO:8201;heparin bindi		Hs.252820	601121	5228
SLC16A3	17q25	GO:15718;	GO:5887;ir	GO:8028;monocarbox		Hs.386678	603877	9123
SCD4	4q21.3			GO:16491;FA_desatui		Hs.379191		79966
SOD2	6q25.3	GO:6979;r	GO:5739;n	GO:8383;manganese		Hs.384944	147460	6648
ST13	22q13.2	GO:6457;p	GO:5737;c	GO:8181;tumor suppr		Hs.377199	606796	6767
TERF2IP	16q23.1	GO:7004;t	GO:781;ch	GO:42162;telomeric D		Hs.274428	605061	54386
TM6SF2	19p13.3-p12					Hs.367829	606563	53345
UQCRB	8q22	GO:9060;a	GO:19866;	GO:8121;u	genmapp_	Hs.131255	191330	7381

SeqDerivedFrom	RefSeq
S67788.1	NM_000038; adenomatosis polyposis coli
NM_006310.1	NM_006310; aminopeptidase puromycin sensitive
NM_004313.1	NM_004313; arrestin beta 2
NM_004536.1	NM_004536; baculoviral IAP repeat-containing 1
X74070.1	NM_001207; basic transcription factor 3
NM_001207.1	NM_001207; basic transcription factor 3
BC002629.1	NM_020993; B-cell CLL/lymphoma 7A
AF015451.1	NM_003879; CASP8 and FADD-like apoptosis regulator
AF009619.1	NM_003879; CASP8 and FADD-like apoptosis regulator
NM_003879.1	NM_003879; CASP8 and FADD-like apoptosis regulator
AF041461.1	NM_003879; CASP8 and FADD-like apoptosis regulator
AF041459.1	NM_003879; CASP8 and FADD-like apoptosis regulator
AL037208	NM_003589; cullin 4A
NM_004720.3	NM_004720; endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4
AF011466.1	NM_004720; endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4
AK025348.1	
AK023938.1	
NM_017932.1	NM_017932; hypothetical protein FLJ20700
AL117451.1	
NM_018041.1	
NM_024984.1	
NM_014709.1	
BC004492.1	NM_014033; DKFZP586A0522 protein
AK001118.1	
NM_018097.1	NM_018097; hypothetical protein FLJ10460
NM_004913.1	NM_004913; chromosome 16 open reading frame 7
AK021514.1	
NM_014339.1	NM_014339; interleukin 17 receptor precursor
AF009634.1	NM_006864; leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domai
AF009644.1	NM_006864; leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domai
NM_006599.1	NM_006599; nuclear factor of activated T-cells 5 isoform c NM_138713; nuclear factor of ac
NM_004855.1	NM_004855; phosphatidylinositol glycan, class B
AK023843.1	NM_002632; placental growth factor, vascular endothelial growth factor-related protein
NM_004207.1	NM_004207; solute carrier family 16 (monocarboxylic acid transporters), member 3
NM_024906.1	NM_024906; hypothetical protein FLJ21032
BF575213	NM_000636; superoxide dismutase 2, mitochondrial
NM_003932.1	NM_003932; heat shock 70kD protein binding protein
NM_018975.1	NM_018975; TRF2-interacting telomeric RAP1 protein
AF130051.1	NM_023002; transmembrane 6 superfamily member 2
NM_006294.1	NM_006294; ubiquinol-cytochrome c reductase binding protein

ns), member 3
ns), member 3
:tivated T-cells 5 isoform b NM_138714; nuclear factor of activated T-cells 5 isoform a NM_173214; nucl

lear factor of activated T-cells 5 isoform a NM_173215; nuclear

Novel Predictors of Cardiac Allograft Rejection Determined by Peripheral Blood Gene Expression

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Conflict of Interest/Disclosure Information

Presenter: Phillip A. Horwitz, MD

Abstract: Novel Predictors of Cardiac Allograft Rejection Determined by Peripheral Blood Gene Expression

Financial Disclosures: None
Unlabeled/Unapproved Use Disclosure: None

Cardiac Allograft Rejection

- 50% of all transplant recipients
- 20% of post-transplant deaths
- Prompt, accurate detection- treatment
- Endomyocardial biopsy to detect cellular rejection- "Gold Standard"
  - Biopsy limitations- sensitivity, cost, invasive, morbidity
- Goal- noninvasive detection of rejection

Acute Rejection Activates Circulating Markers

- T-cell recognition of alloantigens plus co-stimulatory signals
- Cytokine activation
- Graft inflammatory response
- Alteration in circulating leukocyte gene expression levels

Study Hypothesis

- Markers of cardiac allograft rejection can be detected by gene expression profiling in peripheral blood leukocytes using oligonucleotide microarrays

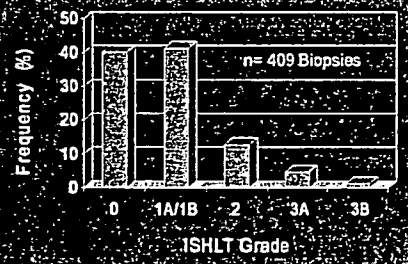
Methods- Sample Collection

- Nested case-control study of peripheral blood specimens
- Endomyocardial biopsy cohort
  - 189 consecutive transplant patients
  - 409 total samples
  - Standard ISHLT criteria
- Sample collection
  - 5cc peripheral blood immediately prior to biopsy
  - Commercial blood RNA storage tubes
  - Stored -80° C : 6+ months

Microarray Screening

- Oligonucleotide microarrays
  - Sample RNA purified
  - Total RNA reverse transcribed cDNA and biotin labeled cRNA
  - Affymetrix HU-133A
  - Hybridized with fluorophore-labeled sample
  - Scanned and quantified for expression level

Endomyocardial Biopsy Cohort



Case-Control Sample Selection

- "Recent" transplant: <18 months
- No overt acute illness
  - Outpatients
  - No active infections
- Stable immunosuppressive regimen
  - Calcineurin inhibitors, anti-metabolites
  - Steroids

Case-Control Sample Selection

- Case patients
  - ISHLT grade 3A or higher rejection
  - 0 or 1 previous episodes of rejection
  - Stored blood samples available pre-, during & post-rejection episode
- Control patients
  - ISHLT grade 0 or 1A rejection
  - No episodes of grade 2 or higher rejection

Methods- Analysis Strategies

1. Case Cross-Over
   - Cases (n=4) vs. Pre/Post Rejection (n=8)
   - Transcripts increase/decreased in rejection biopsy compared with negative (0 or 1A) pre/post biopsies
2. Case-Control
   - Cases (n=4) vs. Controls (n=4)

- 16 total samples for microarray analysis

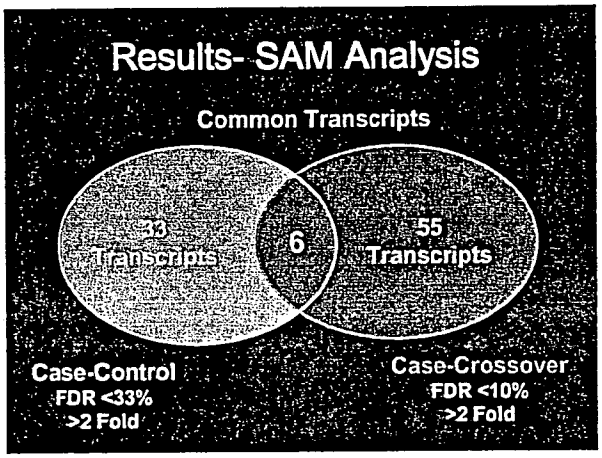
Methods- Data Analysis

- Chip normalization: Robust Multi-array Analysis
  - Background normalization
  - Expression quantification
- Expression comparisons: Significance Analysis of Microarrays (SAM)
  - Fold change
  - False Discovery Rate- multiple comparisons

Results: Case-Crossover Transcripts

- SAM analysis: 55 differentially expressed transcripts (>2 fold, FDR 10%)
- Transcript Classification
  - Immune/ inflammatory responses (31%)
  - Transcription/ translation regulation (20%)
  - Cell signaling pathways (18%)
  - Cell growth and differentiation (11%).

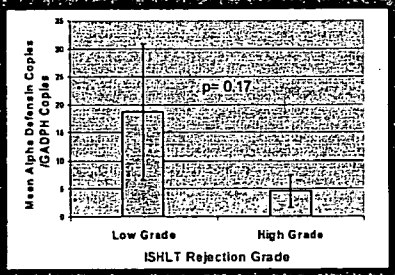
Results- SAM Analysis



Common Transcripts

- Case-Control & Case-Crossover common Transcripts:
  - soluble IL-1 receptor
  - mitochondrial superoxide dismutase
  - ras association domain family
  - TNF alpha-induced protein 2
  - nuclear protein-tara
  - alpha 1-defensin

Quantitative PCR Validation- α-1 Defensin Expression



n= 24 samples

Quantitative PCR from peripheral blood samples

Conclusions

- High quality total RNA successfully obtained from stored peripheral blood leukocytes
- Peripheral blood leukocyte gene expression appears to vary with rejection status
- Novel expression markers associated with rejection identified by microarray analysis

Conclusions II

- Majority of transcripts: immune response, transcription/translation, cell signaling, cell cycle
- Preliminary Validation
  - Initial quantitative PCR data correlates with microarray findings
  - Further validation: additional array samples and quantitative PCR

Collaborators

- Thomas Cappola
- Jonathan Epstein
- John Lepore
- Michael Parmacek
- Andrew Kao
- Shashank Desai
- Lee Goldberg
- Susan Brozena
- Mariell Jessup
- Mary Putt
- Joan Gilmore
- Emily Tsai

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Patient Characteristics

	CASES				CONTROLS			
Age (y)	63	71	44	48	43	63	49	65
Gender	M	F	M	M	M	M	M	M
Graft Age (m)	2.5	8.4	5.7	7.5	2.6	3.3	9.6	16.2
Biopsy	3A	3A	3A	3A	1A	1A	0	0
Prev. Reject	1	1	0	0	0	0	0	0
Immuno-supp	CSA	TAC	CSA	CSA	CSA	CSA	CSA	CSA
Steroid	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

Limitations

- Pilot project- small numbers
- Highly selected case/control samples
- Statistical power/ validity
  - Lack of microarray replicates

Future Directions

- Further define candidate predictors
  - Additional case/control microarrays
  - RNA- Quantitative PCR
  - Correlate with protein, cytokine etc. assay
- Validation cohort
- Gene expression
  - Resolution of rejection, adequacy immunosuppresion, graft-vasculopathy etc.

Methods- Microarray Samples

- RNA Purification
  - Nucleic acid purification column
  - Quality and quantification
    - Gel electrophoresis
    - OD_{260} / OD_{280}
  - 5-15ug total RNA
- Penn Microarray Core Facility
  - Affymetrix HU-133A oligonucleotide microarrays
  - Hybridization, scanning, quantification using standard protocol

Circulating Leukocyte Gene Expression Screening

- Quantitative PCR studies (ex. TNFα, IL-8, IFNγ, granzyme B, perforin, TIRC7)
  - Small numbers of genes
  - Candidate genes identified *a priori*
- Microarray screening
  - Thousands of different genes
  - Can identify novel markers for rejection